

Determining the effect of protozoal inhibitors on protozoal cell concentration, cellular protein, and cell volume to improve livestock feed efficiency

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ABSTRACT: Methane production in ruminants is a large source of inefficiency for producers. Microorganisms that live in the rumen of dairy cattle produce methane as a byproduct of fermentation, and 6% of gross energy cattle ingest is eructated in the form of methane. Rumen protozoa have a close metabolic relationship with methanogenic archaea. Protozoal inhibitors have been developed that target both protozoa within the rumen and their associated methanogens and their suppression may decrease methane production. Essential oils have antimicrobial properties that likely inhibit microbes by disrupting their cell membranes. Unpublished research, conducted by Dr. Ye in the ruminant nutrition laboratory at OSU, investigated the effect of monensin and Cinnagar[®], a product combining the essential oils found in garlic and cinnamon, on protozoa. Results of this research indicated that there was not a decrease in the number of protozoa as expected, but rather there was a decrease in the calculated protozoal nitrogen/cell ratio. The purpose of this study is to further investigate the effects of Cinnagar[®] and monensin on protozoal nitrogen and cell volume using more elaborate laboratory verification. It was hypothesized Cinnagar[®] and monensin would disrupt membrane function and thereby inhibit protozoal metabolic efficiency as measured via the reduction in protozoal volume and mass (protein is the greatest contributor of mass, and protein was assessed using Kjeldahl N), rather than a reduction in the number of protozoa present. This hypothesis was tested by analyzing nitrogen across four treatments and collecting samples for protozoal counts, while simultaneously recording video data for volume determination by video image (by frame) analysis. The four treatments included 1) control (feed only), 2) feed + .0043% DM Cinnagar[®], 3) feed + 2.82 μ M monensin, and 4) feed + Cinnagar[®] + monensin (in the same concentrations). The feed mixture consists of 70% cellulose, 25% potato starch, and 5% glucose. Treatment concentration is consistent with manufacturer feeding recommendations and is the same as concentrations used in the study conducted by D. Ye. Samples were taken at 0, 3, and 6 hours to investigate potential treatment differences across time. Data obtained from Kjeldahl analysis and protozoal counts showed no statistically substantial differences in Nitrogen/ cell between treatments. Data taken from ImageJ analysis of the videos also showed no statistically significant differences in volume of protozoa between treatments. A lower volume and lower standard deviation was seen when comparing the new method of finding volume to the older method used by Clarke³.

Introduction

Ruminants have the ability to convert fiber, unutilized by human digestion, into products humans can utilize, such as milk and meat. Ruminants are able to do this because of the fermentative activity of microbes that inhabit their rumen, or forestomach. This microbial population includes protozoa and bacteria along with lesser activity from fungi, which anaerobically break down fibrous feedstuffs and produce byproducts such as volatile fatty acids that can be utilized by the host animal. Archaea (which resemble bacteria but are in a different domain of life) also use hydrogen and carbon dioxide to produce methane. Microorganisms that pass from the rumen into the stomach also serve as a protein source to the host animal. The role of protozoa in the rumen is still not completely understood, and some studies have suggested that a decreased presence of protozoa in the rumen may increase overall performance of ruminants due to a reduction in the amount of protozoal predation on bacteria (Church, 1988), which are the primary N source for protozoa. In contrast, efforts to effectively suppress protozoa are difficult to apply in commercial settings and can have important negative consequences such as depressed fiber digestibility or milk fat production (Firkins et al., 2007). Methane produced as a byproduct during fermentation in cattle is also of large concern due to its role in global warming (Johnson and Johnson, 1995). More importantly methane production removes carbon that could have otherwise been used as an energy source for the host animal. Using cows in chambers, an average of 6% of gross energy ingested by animals is eructated in the form of methane (Johnson and Johnson, 1995), which contributes substantially to enteric methane production in ruminant livestock operations (Hristov et al., 2013). Ruminal protozoa have a close relationship with methanogens, and their suppression may also decrease methane production, which can decrease feed efficiency; working to decrease methane without losing profit is the ultimate challenge in this area of study. Companies have developed different products to inhibit protozoa within the rumen and associated methanogens. **By better understanding the specific way in which protozoal inhibitors impact protozoa, we can better analyze the effectiveness of these products when fed to ruminants.**

Various research has acquired evidence that Rumensin[®], or monensin (active ingredient) in its basic chemical form, initially inhibits protozoa; however, over time protozoa adapt by changing their membrane structure, decreasing monensin's ability to inhibit them (Karnati et al., 2009). Essential oils are another area of interest resulting from their relative abundance in plants and their antimicrobial properties that likely inhibit microbes by disrupting their cell membranes (Benchaar and Greathead, 2011). Monensin and essential oils have the potential to play a key role in protozoal inhibition and therefore are worth further research and consideration.

Problem Identification and Justification

Previous research has acquired evidence that Rumensin[®] (i.e., monensin is the active ingredient in its basic chemical form) causes protozoa to adapt by changing their membrane structure, decreasing monensin's ability to inhibit them (Karnati et al., 2009). Unpublished research, conducted by D.Ye in the ruminant nutrition laboratory at OSU, investigated the effect of monensin and Cinnagar[®], which is a product combining the essential oils found in garlic and cinnamon, on protozoa either not supplemented or supplemented with monensin, Cinnagar[®], or their combination, in a batch culture experiment using continuous culture fermenters. They

hypothesized that the combination of monensin and Cinnagar[®] would more effectively inhibit protozoa, in turn increasing rumen efficiency and decreasing methane production (Firkins et al., 2012). Results of this research, attained by protozoal enumeration alongside nitrogen analysis, indicated that there was not a decrease in the number of protozoa as expected, but rather there was a decrease in the protozoal nitrogen/cell ratio (Firkins, et al. 2012). Doing further research to better understand why these results occurred will help to better understand how these inhibitors affect protozoa.

In order for the nitrogen per cell ratio to decrease without decreasing total number of protozoa, we suspect that protozoal inhibitors primarily inhibit protozoa by decreasing cell volume or by decreasing protein concentration per cell. Being able to compare a more accurate average protozoal volume attained from video analysis alongside protein or nitrogen values will also increase understanding of these protozoal inhibitors. Developing a method of taking video opens up possibilities of measuring cell membrane translucence, tracking motility, and measuring volume the combination of which will allow for overall better understanding of how monensin and Cinnagar[®] work together to influence fermentation stoichiometry.

Hypothesis and Objectives

It was hypothesized that rumen protozoa would be significantly inhibited by Cinnagar[®] and monensin via the reduction in protozoal volume and altering of the cell membrane, rather than a reduction in the number of protozoa present. This hypothesis was tested by analyzing protozoal nitrogen across 4 treatments and collecting samples for protozoal counts, while simultaneously recording video data for volume determination by video analysis.

Objective 1: Develop a method to apply treatments, record video data of live protozoa, and later isolate protozoa from sampled rumen fluid to permit measurement of protozoal nitrogen concentration.

Objective 2: Compare nitrogen/cell ratio of isolated protozoa across treatments; use video data to analyze volume per protozoa.

Methods and Materials

Objective 1: Develop method to apply treatments, record video data of live protozoa, and later isolate protozoa from sampled rumen fluid to permit measurement of protozoal nitrogen.

Rumen fluid from cannulated dairy cows is routinely collected and used for various analyses within our lab. Flootation of small particulates that are buoyed by gas production (“flocculation”) and filtering to remove feed from rumen fluid is often necessary to obtain cleaner samples and thereby reduce nitrogen contamination. I will further develop methods of feeding and filtering specific to my needs.

Thus far we have developed a filtering method largely based on research done by John Sylvester, in which filter bags are used to remove bacteria and smaller feed particles from samples, leaving behind isolated protozoa with less contamination (Sylvester, et al., 2004). Samples were placed in a 10-micron filter bag and washed 7 times in 0.9% saline or until the saline remains clear. This method of washing was used in Objective 2 to produce cleaner samples for determination of protozoal nitrogen.

Using a procedure developed for taking video of live protozoa, I will document a representative sample (total of 9 videos, 10 seconds each) for each treatment at each desired incubation time. These videography methods were used in Objective 2 to evaluate treatment effect on protozoal volume.

Objective 2: Compare nitrogen content of isolated protozoa across treatments; use video data to analyze volume per protozoa and average motility.

Rumen fluid was collected from cannulated lactating dairy cows at Waterman Dairy Center and then flocculated with simplex buffer (1:1, rumen fluid: simplex buffer) to remove feed using natural microbial fermentation action. After removing as much feed as possible via flocculation and aspiration, 30-mL of inoculate was distributed anaerobically into tubes containing appropriate treatments. The four treatments include: 1) control (feed only), 2) feed + .0043% DM Cinnagar[®], 3) feed + 2.82 μ M monensin, and 4) feed + Cinnagar[®] + monensin (in the same concentrations; Figure 1). Treatment concentration is consistent with manufacturer feeding recommendations and is the same as concentrations used in the study conducted by D.Ye. The feed mixture added, 0.5g of which was added to each tube, consisted of 70% cellulose, 25% potato starch, and 5% glucose. Feeding is necessary because these treatments are given as a percentage of the diet. This specific feed mixture was chosen because it does not contain nitrogen and therefore ensures that all nitrogen measured are of microbial origin during analysis. The experiment consisted of 4 treatments and 3 timepoints at 0, 3, and 6 hours, each done in duplicate, for a total of 24 tubes per replication for 2 replications.

Figure 1: Treatment Description

Control (1)	Cinnagar (2)	Monensin (3)	Cinnagar+Monensin (4)
<ul style="list-style-type: none"> • 0.5g of feed mixture* • 100% EtOH added to account for dilution in (3) and (4) 	<ul style="list-style-type: none"> • 0.5g of Cinnagar[®] treated feed mixture* (0.0043% Cinnagar[®]) • 100% EtOH added to account for dilution in (3) and (4) 	<ul style="list-style-type: none"> • 0.5g of feed mixture • 2.82 μM monensin dissolved in 100% EtOH 	<ul style="list-style-type: none"> • 0.5g of Cinnagar[®] treated feed mixture* (0.0043% Cinnagar[®]) • 2.82 μM monensin dissolved in 100% EtOH
<p>*All tubes were given 0.5 g of 70% cellulose, 25% potato starch, and 5% glucose. Cinnagar was mixed in with feed provided to treatments (2) and (4). All tubes were inoculated with 30 mL of 50% rumen fluid.</p>			

At 0, 3, and 6 hr after ruminal inoculum was added, 0.5 mL of sample was taken for video analysis, and the remaining volume fixed in formalin for future filtering and counting. After filtering, a Kjeldahl analysis was performed on the contents of the bag (isolated protozoa) to determine total nitrogen concentration of remaining protozoa.

Video data were analyzed using ImageJ¹¹ to determine average volume and motility. These values were compared across treatments and time points to determine overall treatment affect and to test for potential treatment interactions with time. ImageJ¹¹ is typically used with fixed cells; cell fixation can modify cell volume and therefore can give data that are not representative of true volume before fixation. Fixing cells before data collection also eliminates our ability to look at motility factors compared with structure characteristics. By developing a method to take video and modifying ImageJ¹¹ data analysis using live protozoa countless possibilities of data and comparisons, such as motility to cell structure, are made possible.

Results

Data obtained from Kjeldahl analysis and protozoal counts showed no statistically substantial differences in Nitrogen/ cell between treatments. Data taken from ImageJ analysis of the videos, using PROC MIXED from SAS/STAT[®] software^{10,15}, also showed no statistically significant differences in volume of protozoa between treatments. A lower volume and lower standard deviation was seen when comparing the new method of finding volume to the older method used by Clarke³ (Fig. 2).

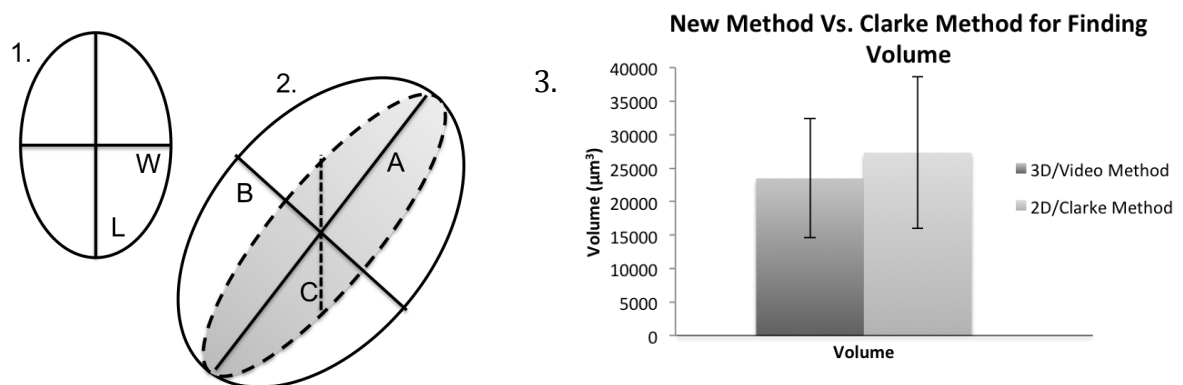


Fig. 2: 1. Clarke's method of volume determination using photographs ($\text{Volume} = 0.45(LW^2)$); 2. New method of volume determination using videography ($\text{Volume} = 4/3 (ACB)$); 3. Comparison of two methods, $P < 0.01$

Conclusions and Implications

Statistically this data can neither support nor reject the null hypothesis. More replications need to be performed to get more conclusive results. Ultimately the method of video analysis for finding protozoal volume was proven as a viable option for future studies, as well as more practical and accurate than older methods. These video techniques have the potential to increase our

understanding of specifically how protozoa are inhibited using feed additives, such as Rumensin[®] and Cinnagar[®].

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